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Regeneration of the Pharynx in a Freshwater Planarian: An Electron-Microscopic Study with Special Reference to the Formation of the Pharyngeal Cavity and Pharyngeal Lumen

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ABSTRACT—Formation of the pharynx, with special reference to the formation of the pharyngeal cavity and pharyngeal lumen, was studied by electron microscopy during regeneration of head fragments of planarians transected at the prepharyngeal region. A slit occurs on the third-day at the proximal region of the blastema, which is then enlarged to become the pharyngeal cavity. Another slit is formed on the fourth day at the boundary between the proximal part of the pharyngeal rudiment and the closed end of the cut main-intestinal trunk, and this slit is later enlarged to penetrate into the pharyngeal rudiment, subsequently becoming the pharyngeal lumen. A common ultrastructural feature of cells that participate in the formation of these slits is the presence of rod-shaped bodies and microvilli. A few slits of small size in the tissue are often seen separate from the rudimental pharyngeal cavity. These slits become connected with one another and, consequently, the rudimental pharyngeal cavity develops into a larger cavity. Formation of phagosomes and disintegration of cells are seen as common morphological features of the processes of enlargement of the pharyngeal cavity and lumen, and it is deduced that such disintegration plays a significant role in the enlargement of the open space. The intestinal cells near the cut end of the intestine separate from the intestinal tissue and take part in the recruitment of epithelial cells facing the pharyngeal lumen. From these observation, transdifferentiation of separated intestinal cells is ascertained.

INTRODUCTION

Since Morgan [1] pointed out that two phenomena, namely epimorphosis and morphallaxis, are the most important processes of animal regeneration, studies on mechanisms of regeneration have centered on attempts to explain these two phenomena. With respect to epimorphosis, many studies have been carried out on the regeneration of amphibian limbs and, consequently, it has been confirmed that lost parts are repaired by the dedifferentiation of cells that are already present, by proliferation of cells, and by redifferentiation. However, in the case of regeneration of planaria, some phenomena that are impossible to explain by epimorphosis alone have been observed; during the processes by which small pieces of planaria regenerate to form complete animals with normal proportions, changes in the positions of original cells and reorganization of body tissues, including cell transdifferentiation are considered to take place. In order to know the mechanism of regeneration of planaria, therefore, it is absolutely necessary to clarify the process of morphallaxis. Since the formation of the pharynx in planarian regeneration is carried out mainly by typical morphallaxis [2], this system seems to have potential for studies to clarify its mechanism.

Some histological investigations have been carried out on the regeneration of the pharynx [3–9]. However, since such studies have generally emphasized the origin and behavior of formative cells, little attention has been paid to the fact that formation of the pharynx is carried out by morphallaxis, which includes the transdifferentiation of cells. Unfortunately, observations by light microscopy are technically inadequate for clarifica-

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tion of phenomena such as cellular dedifferentiation and transdifferentiation, that is, electron microscopy is indispensable for observations of such phenomena. Nevertheless, to date, no electron-microscopic study of the formation of the pharynx has been performed. In the present study, electron-microscopic observations were made with a view to clarifying the differentiation of cells during formation of the pharynx, in particular during the early stages of formation of both the pharyngeal cavity and the pharyngeal lumen which, in due course, combine to form the cylindrical pharynx.

MATERIALS AND METHODS

The material used in this study was the sexual form of the freshwater planarian, *Dugesia japonica japonica.* After one week or more of starvation, the worms, 8 to 10 mm in length, were transected at the prepharyngeal region and the anterior pieces were allowed to regenerate in an incubator at $18 \pm 1^{\circ}C$ in water taken from their native brook. For preliminary observations by light microscopy, groups of five regenerates each were fixed every day over a period of 15 days after transection. The fixed specimens were dehydrated with a graded ethanol series, embedded in Paraplast, sectioned sagittally or horizontally at 6 μm, and stained with Mayer's hemalum and eosin B. For electron microscopy, the intact worms and the regenerates were fixed for 60 min at 4°C in 2.5% glutaraldehyde, which had been buffered at pH 7.4 with 0.1 M cacodylate buffer, and then post-fixed in 2% osmium tetroxide in the same buffer for 90 min at 4°C. After dehydration in a graded ethanol series, the specimens were embedded in Epon 812 or Quetol 812. Thick sections, 0.5–1 μm thick, stained with 1% toluidine blue, were also examined by light microscopy. Thin sections were cut on a Porter-Blum MT-2B ultramicrotome with a diamond knife. After staining with uranyl acetate and lead citrate, sections were observed under a Hitachi H-500 (100 KV) electron microscope at magnifications from $\times 2{,}500$ to $\times 50{,}000$.

RESULTS

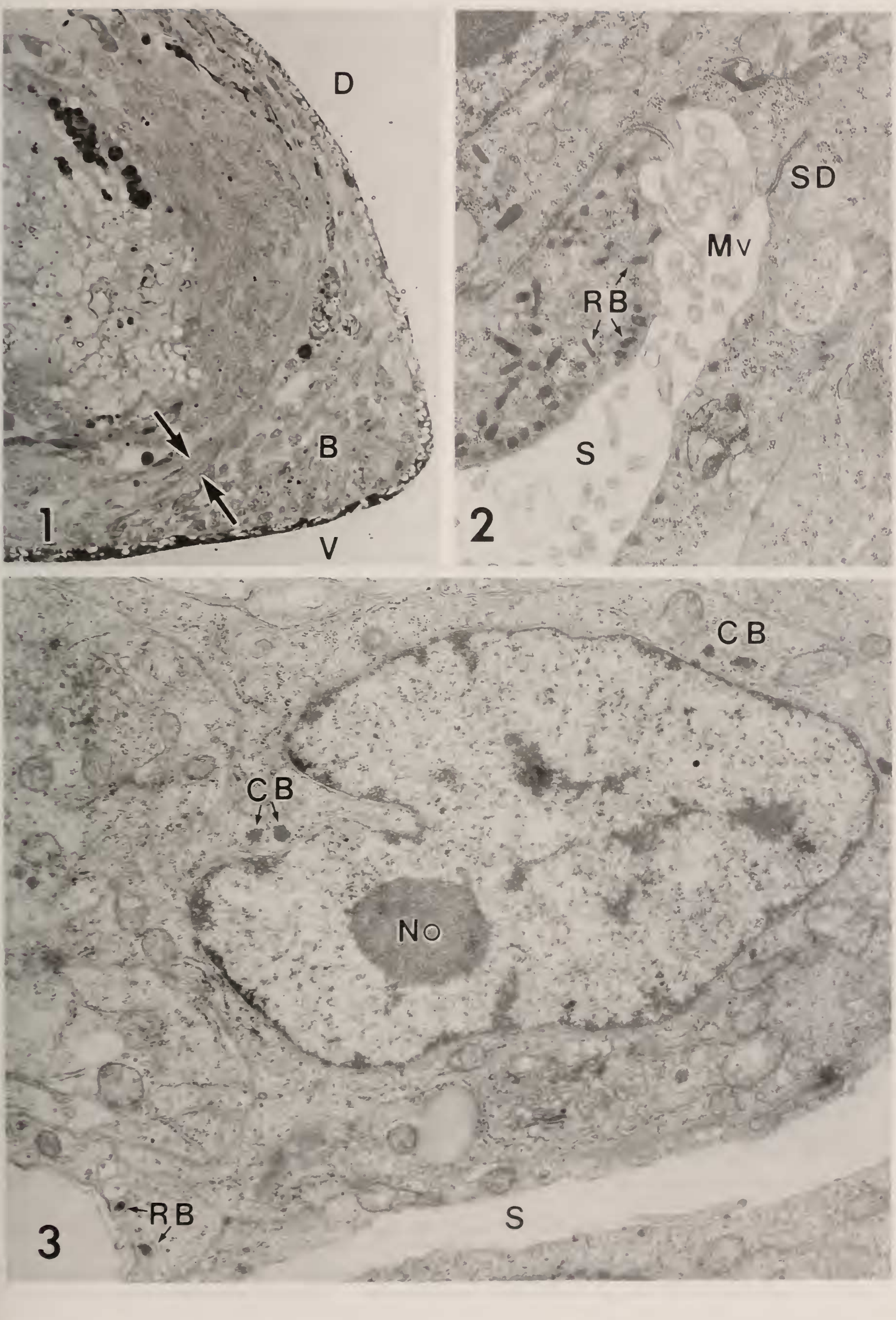
In semi-thin sections of second-day regenerates derived from the anterior pieces of worms transected at the prepharyngeal region, the blastema appears as just an accumulation of undifferentiated cells under the light microscope. In third-day regenerates, an extremely narrow slit is seen in the blastema (Fig. 1). The slit is formed mainly at the ventral region of the proximal part of the blastema and also, albeit rarely, at the dorsal or distal parts of the blastema. This slit is the first visible sign indicative of formation of the pharynx and is a rudiment of the pharyngeal cavity.

Electron-microscopic observations of the cells that face the slit reveal that they are connected with one another by septate desmosomes, and each of them has many microvilli on its free surface and contains electron-dense granules in the cytoplasm near the free surface (Fig. 2). These granules are globular or ellipsoidal, about 50–250 nm in diameter, and they are enclosed by a membrane. They can be regarded as immature versions of the rod-shaped bodies that are characteristic in intact pharyngeal epithelial cells. Such cells also contain mitochondria with well-developed cristae, small vesicles, lipid droplets, and microtubules. However, in the cytoplasm near the nuclear envelope, chromatoid bodies characteristic of undifferentiated cells, i.e., neoblasts, can be seen (Fig. 3). The cells covering the anterior part of the slit eventually form the pharyngeal outer epithelium, while those covering the posterior part become the epithelium of the pharyngeal atrium. There are distinct differences between these two

Fig. 1. A light micrograph showing the initiation of formation of the pharynx in a third-day regenerate. The slit (arrows) is a rudiment of the pharyngeal cavity and is formed mainly at the ventral region of the proximal part of the blastema (B). D: dorsal side. V: ventral side. $\times 300$.

Fig. 2. An electron micrograph of the cells that face the slit (S) in a third-day regenerate. They are connected with one another by septate desmosomes (SD), and each of them has many microvilli (Mv) and contains rod-shaped bodies (RB). $\times 19{,}500$.

Fig. 3. The cells that face the slit (S) in a third-day regenerate. The cells contain chromatoid bodies (CB) characteristic of undifferentiated cells. RB: rod-shaped bodies. No: nucleolus. $\times 15{,}400$.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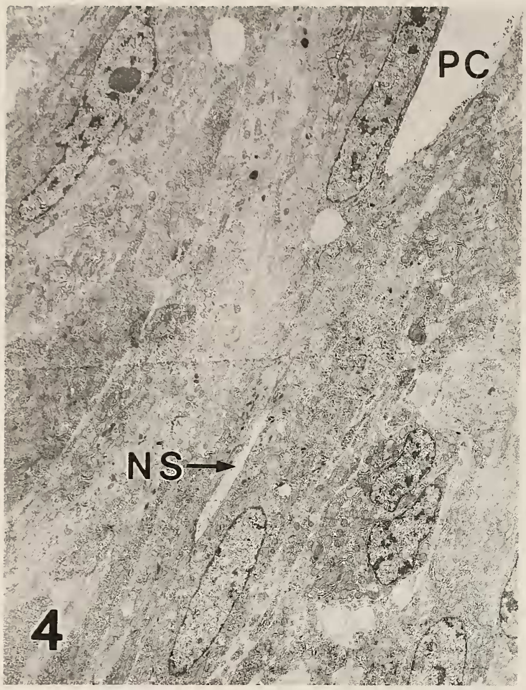
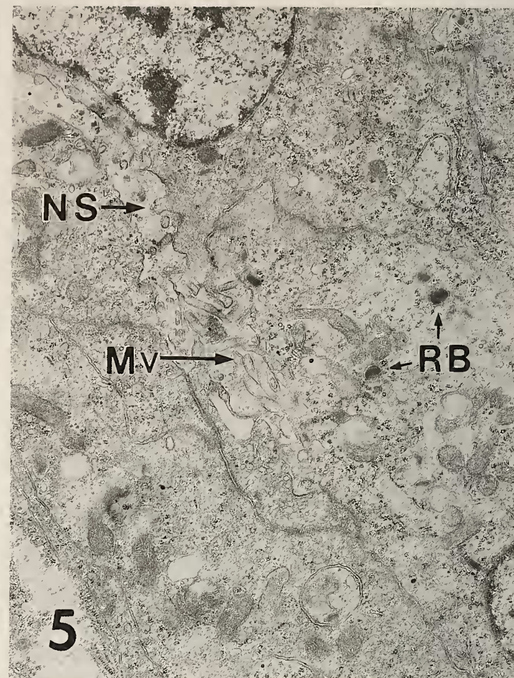
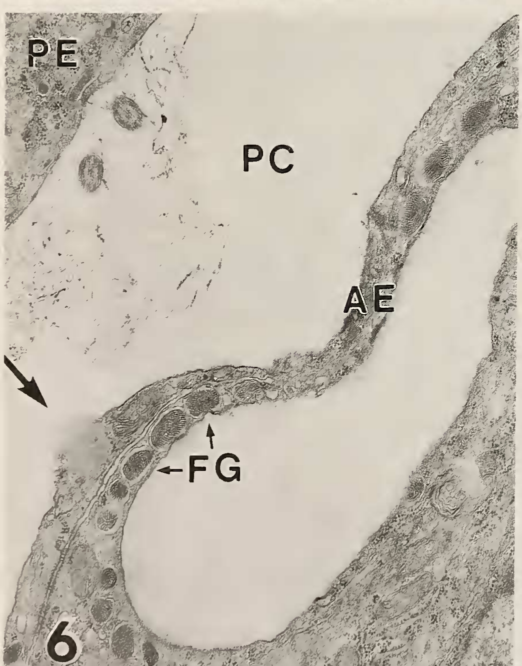






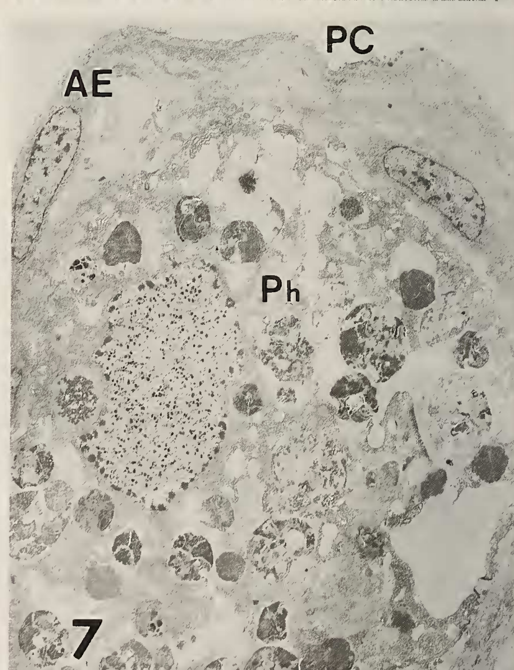


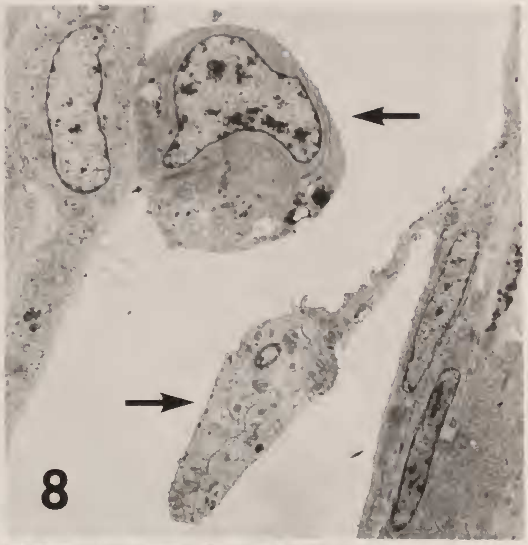
Fig. 4. A newly formed slit (NS) in a sixth-day regenerate. The narrow slits are aligned to extend from the ends of the pharyngeal cavity rudiment (PC). ×3,500.

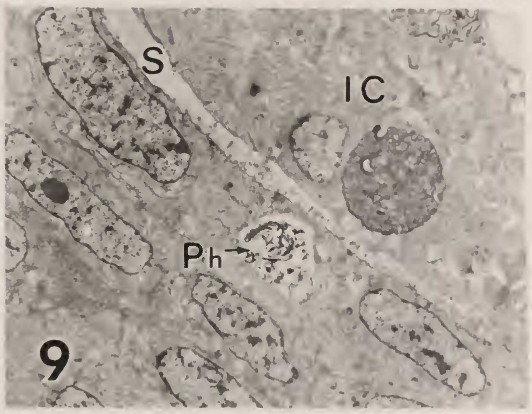
Fig. 5. The cells facing a newly formed slit (NS) in a sixth-day regenerate contain rod-shaped bodies (RB) and have microvilli (Mv). ×10,000.

Fig. 6. The photograph of a sixth-day regenerates showing that the fingerprint-like granules (FG) are released into the pharyngeal cavity (PC) together with part of the cytoplasm. A large arrow indicates disintegration of the

types of cell at their final stage of differentiation. The most significant difference is that the former cells contain rod-shaped bodies, while the latter cells contain none but, instead, they contain granules with a fingerprint-like structure. At the early stage of formation of the pharynx, however, cells of both types contain only rod-shaped bodies (Figs. 2, 3). This observation suggests that they originate from the same kind of cell. The blastema including the cavity rudiment is composed not only of undifferentiated cells, but also of various types of cell, such as cells with secretory granules, neurosecretory granules, rhabdites, myofilaments, or rod-shaped bodies. A few narrow slits are often seen, distributed in the blastema tissue, a little apart from the ends of the rudiment of the pharyngeal cavity (Fig. 4). The cells facing these newly formed slits also contain rod-shaped bodies in their cytoplasm near the free surface and have microvilli on their free surfaces; even before a slit is formed between cells, these structures are seen in the now-adhering, but soon-to-separate, surfaces of adjacent cells (Fig. 5). These slits are connected with the early-stage pharyngeal cavity; they grow larger and eventually form the complete pharyngeal cavity. Therefore, it seems that microvilli and rod-shaped bodies play a crucial role in this process of formation of slits and development of the pharyngeal cavity.

As expansion of the pharyngeal cavity proceeds, well-defined changes occur in the presumptive epithelial cells of the pharyngeal atrium. The fingerprint-like granules, characteristic of this type of cell, take the place of the rod-shaped bodies that





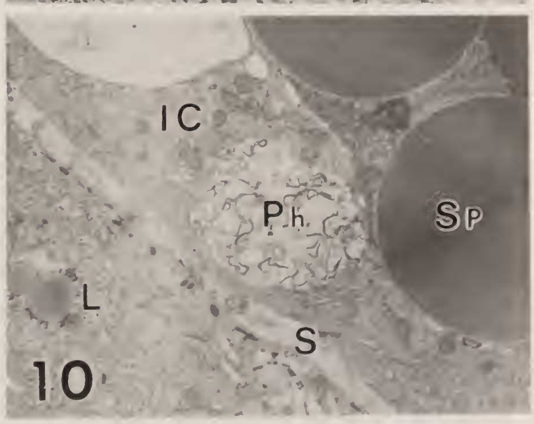


FIG. 8. Separated masses (arrows) seen in a fifth-day regenerate. They were often released into the pharyngeal cavity (PC). ×4,000.

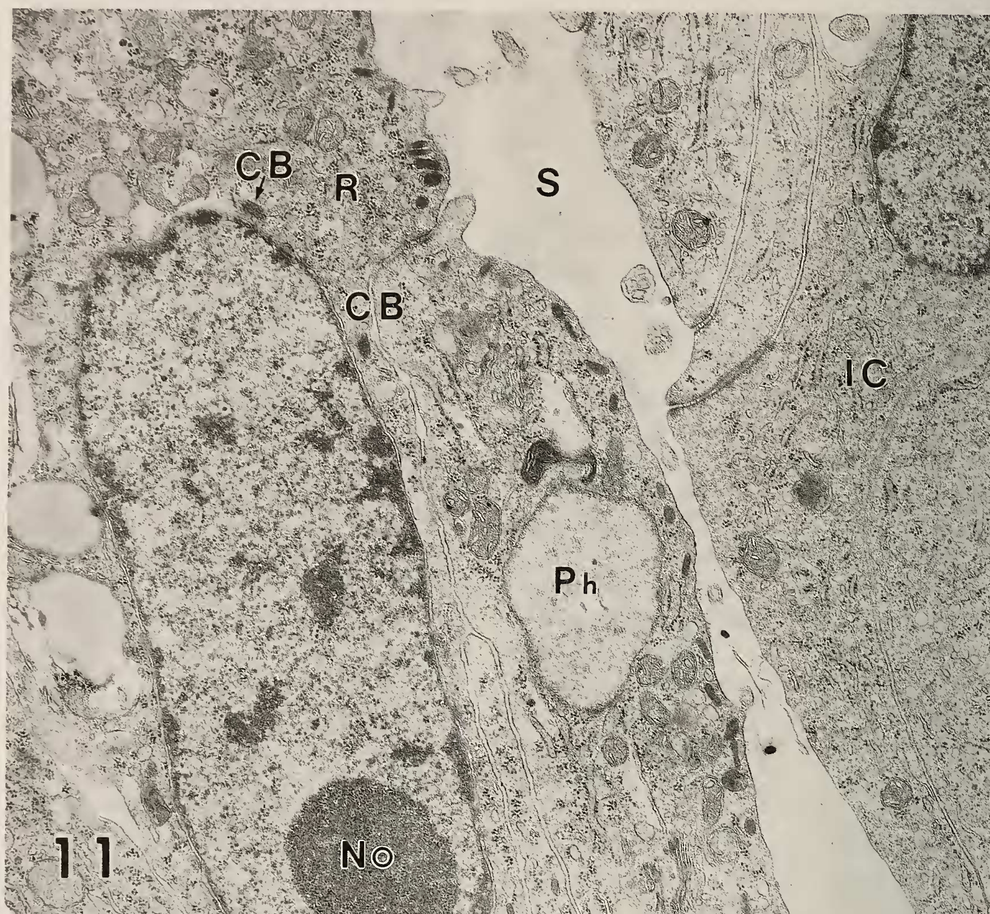
FIG. 9. A slit (S) which is a rudiment of pharyngeal lumen in a fourth-day regenerate. IC: intestinal cell. Ph: phagosome. ×4,000.

FIG. 10. The intestinal cell (IC) forming the anterior wall of the slit (S) in a fourth-day regenerate. L: lipid droplet. Sp: spherical granules. Ph: phagosome. ×8,000.

cytoplasm. AE: epithelium of pharyngeal atrium. PE: pharyngeal epithelium. ×18,700.

FIG. 7. Huge phagosomes (Ph) are seen in the posterior region of the pharyngeal cavity (PC) in a fifth-day regenerate. AE: epithelium of the pharyngeal atrium. ×2,500.

were formerly present. The fingerprint-like granules are released into the pharyngeal cavity together with a part of the cytoplasm (Fig. 6), and it seems that such secretion of the apocrine type also contributes to the expansion of the pharyngeal cavity. It was revealed that the cells at the



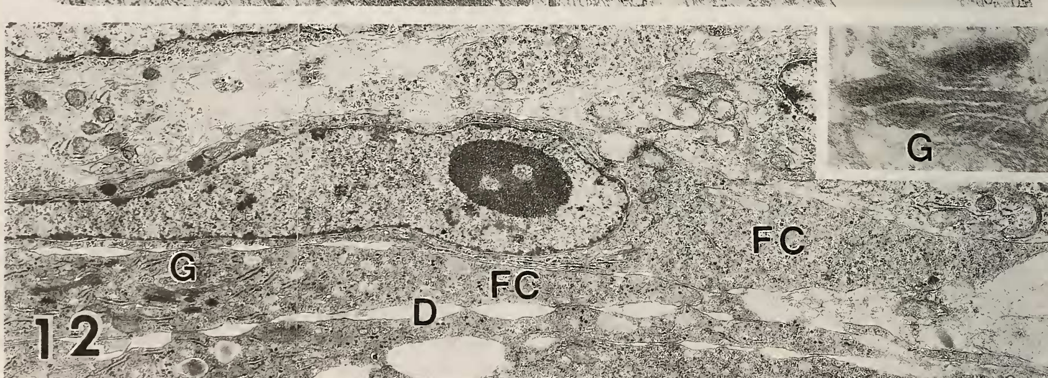
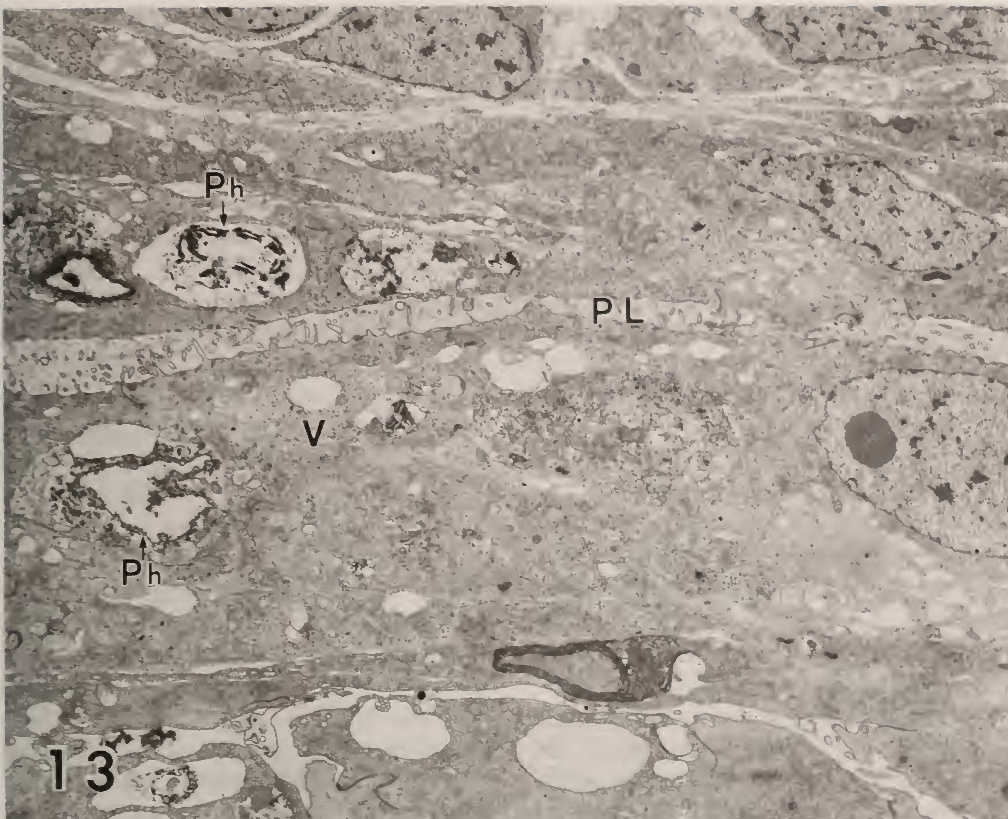
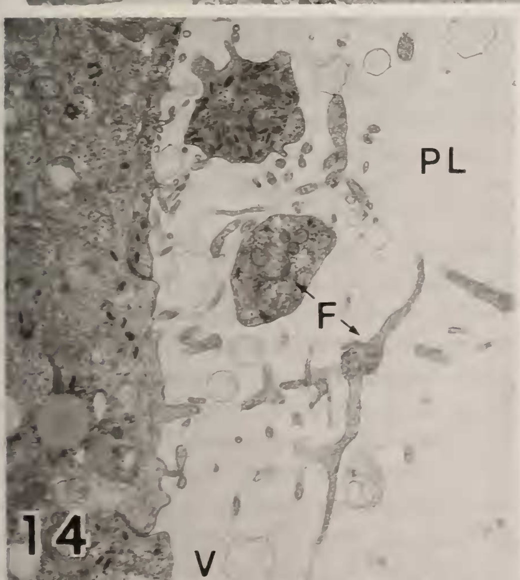


Fig. 11. The cells forming the posterior wall of the slit (S) in a fourth-day regenerate. CB: chromatoid bodies. IC: intestinal cell. No: nucleolus. Ph: phagosome. R: ribosomes. ×17,300.

Fig. 12. The free cells (FC) which have left the basal lamina retain their characteristics of intestinal cells in a fifth-day regenerate. ×9,000. Inset: Golgi lamellae (G) with a feather-like structure. ×45,000.





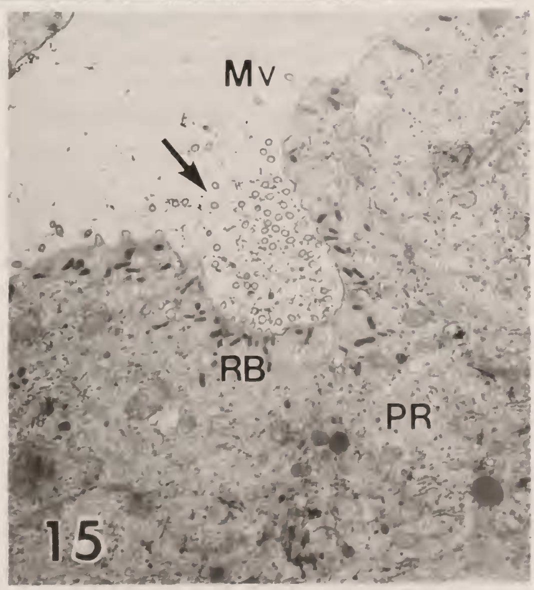


FIG. 13. The cells that compose the rudimentary pharyngeal lumen (PL) in a fifth-day regenerate. Ph: phagosome V: vacuole. ×5,500.

FIG. 14. Disintegrated fragments (F) of cytoplasm and vacuoles (V) seen in the pharyngeal lumen (PL) in a fifth-day regenerate. ×8,000.

FIG. 15. An invagination or pit (arrow) formed at the median part of the posterior distal tip of the pharyngeal rudiment (PR) in a sixth-day regenerate. Mv: microvilli. RB: rod-shaped bodies. ×9,300.

posterior part of the pharyngeal cavity often contained phagosomes (Fig. 7) and that disintegrating cells were often released into the pharyngeal cavity (Fig. 8). Therefore, this disintegration of cells seems also to play a significant role in the expansion of the pharyngeal cavity.

The parenchyma of the pharyngeal rudiment is formed by the gathering together of cells that have migrated from the anterior region of the regenerate. The formation of the pharyngeal lumen starts already on the fourth day with formation of a long and narrow intercellular space at the boundary between the closed end of the cut main-intestinal trunk and the proximal part of the pharyngeal rudiment (Fig. 9). The cells forming the anterior wall of the lumen rudiment have ultrastructural features characteristic of intestinal cells, such as large spherical granules, well-developed rough-surfaced ER, phagosomes, and lipid droplets (Fig. 10). By contrast, the cells forming the posterior wall of the slit have many microvilli that protrude into the space and contain rod-shaped bodies characteristic of pharyngeal epithelial cells, and they are attached to neighboring cells by septate desmosomes. However, they also possess characteristics peculiar to undifferentiated cells, such as a large nucleolus, chromatoid bodies, and many free ribosomes (Fig. 11). In addition, they also possess some of the characteristics of intestinal cells, such as phagosomes, rough-surfaced ER, and lipid droplets. On the 4.5th day, the intestinal cells situated a little anterior to the cut end of the main-intestinal trunk are always observed to be extended, as long and slender fingers, toward the cut end; these cells are situated on the basal lamina that surrounds the intestinal tissue, though the extent of their areas of contact with the lamina is extremely limited. Thus, it seems very easy for such cells to leave the basal lamina and become free cells. In fact, on the fifth day, the free cells crowding the lateral sides of the rudiment of the pharyngeal lumen retain their characteristics of intestinal cells, such as Golgi lamellae with feather-like structures, with dilated intercellular spaces (Fig. 12) in addition to rod-shaped bodies. This morphology indicates that the free intestinal cells probably take part in the recruitment of epithelial cells of the pharyngeal lumen. Phagosomes and vacuoles are often observed in the cytoplasm of the cells that compose the pharyngeal lumen at this stage (Fig. 13), and disintegrated fragments of cytoplasm are seen to be abundant in the lumen space (Fig. 14). On the sixth day, an invagination or pit is observed at the median part of the posterior distal tip of the pharyngeal rudiment (Fig. 15). From the pit, an enlargement of the intercellular spaces between pharyngeal epithelial cells is seen to spread, moving forward in the anterior direction. The cells facing the pharyngeal cavity, with rod-shaped bodies in the cytoplasm near the surface and with microvilli that protrude into the space, release a lot of fibrous material. Consequently, the invagination is connected with the rudimental pharyngeal lumen, completing the pharyngeal lumen.

DISCUSSION

The pharynx of the triclad planarian is cylindrical in shape, and it is composed of complicated layers of tissues. When such a complicated pharynx is being built up in a blastema, which is just a disorganized group of various kinds of cell, it is to be expected that various morphogenetic phenomena take place in cooperation with one another in order to complete the process of formation of the pharynx. An understanding of this process will provide not only an explanation of the mechanisms of morphallaxis in regenerative phenomena, but also a clue to the explanation of cellular behavior in general developmental phenomena.

In regeneration of the pharynx, the formation of the contours of a cylindrical form depends on the formation of the pharyngeal cavity which separates the pharynx parenchyma from the rest of the body tissues, and also on the formation of the pharyngeal lumen which penetrates through the pharyngeal parenchyma. Therefore, the formation of the cavity and the lumen can be regarded as the most fundamental aspect of the morphogenesis of the pharynx. Kido [10–12] studied the process of formation of the pharynx in *Dugesia gonocephala* (=*D. japonica japonica*) by light microscopy, and maintained that the epithelial cells that form the pharyngeal lumen originate from the cells that have isolated themselves from the cut ends of intestinal tissue. Subsequently, Ichikawa and Ishii [13] and Teshirogi [14] also suggested the same phenomenon from their studies of *Dendrocoelopsis lacteus* and *Bdellocephala brunnea* respectively. However, they did not study behavior and transdifferentiation of the cells participating in the process of the formation of pharyngeal cavity and lumen. The present study examined this problem by electron microscopy in *D. japonica japonica*.

From many light-microscopic studies of planarian blastemas, it was assumed that the blastema might be a pile of neoblasts exclusively [7, 15, 16]. However, electron-microscopic observations revealed that the blastema is composed of a heterogeneous population of cells [17, 18]. In the present study also, it is clear that the blastema is composed not only of neoblasts but also of cells with various differentiated characteristics. The cells that undergo transdifferentiation most rapidly and manifest differentiated characteristics in such a heterogeneous mass of cells at the early stage of regeneration are expected to be intestinal epithelial cells, considering that a number of cells which have separated from the intestine were observed to contain rod-shaped bodies characteristic to pharyngeal epithelial cells. Then, these cells form an aggregate of cells via intercellular adhesion of cells of the same type [19], and the formation of the pharyngeal cavity starts in the aggregated cluster of free cells from the intestine. A similar phenomenon was reported in the case of formation of the eye in regeneration of the planarian head [20, 21]; two types of cell, those which originate from nerve-cord cells and primitive pigment-forming cells, reaggregate and establish the fundamental form of the eye by the segregation of cells. Accordingly, it can be considered that in organogenesis in planarian regeneration, the following processes generally occur: some of the cells of the remaining body tissues transdifferentiate, and then they seggregate and form the prescribed organs.

The present study, moreover, showed that the enlargement of intercellular spaces occurred between cells in which rod-shaped bodies are formed, and then the enlargement of the space proceeded further via protrusion of microvilli and, consequently, the rudiment of the pharyngeal cavity was formed. What causes the enlargement of the intercellular space between the epithelial cells? Although no confirmation of this possibility exists to date, it is possible that the materials of the rod-shaped bodies are secreted and dissolve the intercellular adhesive substances. This possibility is strengthened by the observation that rod-shaped bodies are always arranged in a line near the adjacent but soon-to-be-separate surfaces of cells in contact. These morphological aspects of the enlargement of the intercellular space were always observed in all cases of space formation of the cavity, lumen and tissue slit. How do the early-stage slits in the tissue expand to become a completed pharyngeal cavity or a pharyngeal lumen? The results of the present study indicate that both cavity and lumen are formed by fundamentally the same process. Namely, the expansion of the enlarged intercellular spaces occurs via a series of connections of the narrow tissue slits. At the same time, the expansion proceeds also via cellular disintegration and the release of disintegrated cytoplasmic materials into the space. Although cellular disintegration is a phenomenon ordinarily seen in regeneration processes, in this case the cellular disintegration, which takes place at random, does not make any positive contribution to this process, but it appears that the process of formation of the space causes some cells to disintegrate. Probably, this process is similar to the phenomenon of disintegration of specific clusters of cells in the formation of fingers in vertebrates [22].

In planarians, it is well known that, when a head piece is transplanted into the postpharyngeal region, two new additional pharynges are induced at sites anterior and posterior to the graft [23–25]. The author ascertained previously, by labeling of cells with a radioisotope and X-irradiation, that the cells which make up induced pharynges originate from the tissue cells of the host [26, 27]. In this case, does cell transdifferentiation occur in the intercalary regeneration between host and graft [24, 28]? This question seems to be related to the strong morphallactic capacity of this worm, and it appears worthy of further study in the future

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